Oligodeoxynucleotide CpG 7909 Delivered as Intravenous Infusion Demonstrates Immunologic Modulation in Patients With Previously Treated Non-Hodgkin Lymphoma

Brian K. Link,† Zuhair K. Ballas,*†‡ Daniel Weisdorf,§ James E. Wooldridge,*† Aaron D. Bossler,¶ Mary Shannon,† Wendy L. Rasmussen,* Arthur M. Krieg,‖ and George J. Weiner*†*

Summary: Oligodeoxynucleotides containing CpG motifs (CpG ODN) can alter various immune cell subsets important in antibody therapy of malignancy. We undertook a phase I trial of CPG 7909 (also known as PF-3512676) in patients with previously treated lymphoma with the primary objective of evaluating safety across a range of doses, and secondary objectives of evaluating immunomodulatory effects and clinical effects. Twenty-three patients with previously treated non-Hodgkin lymphoma received up to 3 weekly 2-hour intravenous (IV) infusions of CPG ODN 7909 at dose levels 0.01 to 0.64 mg/kg. Evaluation of immunologic parameters and clinical endpoints occurred for 6 weeks. Infusion-related toxicity included grade 1 nausea, hypotension, and IV catheter discomfort. Serious adverse hematologic events observed more than once included anemia (2 = Gr3, 2 = Gr4), thrombocytopenia (4 = Gr3), and neutropenia (2 = Gr3), and were largely judged owing to progressive disease. Immunologic observations included: (1) The mean ratio of NK-cell concentrations compared with pretreatment at day 2 was 1.44 (95% CI = 0.94-1.94) and at day 42 was 1.53 (95% CI = 1.14-1.91); (2) NK activity generally increased in subjects; and (3) Antibody-dependent cellular cytotoxicity activity increased in select cohorts. No clinical responses were documented radiographically at day 42. Two subjects demonstrated late response. We conclude CpG 7909 can be safely given as a 2-hour IV infusion to patients with previously treated non-Hodgkin lymphoma at doses that have immunomodulatory effects.

Received for publication November 21, 2005; accepted February 14, 2006.

From the Departments of *Internal Medicine; ¶Pathology; †Holden Comprehensive Cancer Center, University of Iowa; ‡Iowa City Veteran's Affairs Medical Center, Iowa City, IA; §University of Minnesota, Minneapolis, MN; and ‖Coley Pharmaceutical Group, Wellesley, MA.

Supported by General Clinical Research Center Grants NIH RR00059, NCI-P50 CA97274 and a grant from Coley Pharmaceutical Group. Arthur M. Krieg is employed by Coley Pharmaceutical Group.

Reprints: George J. Weiner, MD, University of Iowa, 200 Hawkins Drive, 5970Z JPP, Iowa City, IA 52242 (e-mail: george-weiner@uiowa.edu).

Key Words: cancer immunotherapy, lymphoma, CpG ODN, TLR agonist, phase I trial

(*J Immunother* 2006;29:558–568)

Non-Hodgkin lymphoma (NHL) frequently responds to immune modulation as indicated by clinical responses to interferon (IFN), interleukin (IL)-2, and anti–B-cell monoclonal antibodies (moabs).[1–4] Moabs probably work through a variety of mechanisms, including antibody-dependent cellular cytotoxicity (ADCC).[5–7] Speculation regarding the effector cells that mediate ADCC centers on macrophages, monocytes, and NK cells.[8,9] Recent data demonstrating that CD16 genetic polymorphisms predict for response to moab therapy provide further evidence that NK or other CD16 (+) cells play an important role in the antitumor activity of moabs.[8] Recent studies suggest that presence of an "immune effector" profile on microarrays of gene expression predicts for a higher likelihood of response to rituximab in follicular lymphoma.[10] Therefore, strategies to enhance the activation of immune effector subsets, including NK cells, may be useful in augmenting the clinical efficacy of moab therapy.

Oligodeoxynucleotides (ODNs) are short sequences of chemically modified deoxyribonucleic acids with sequence-specific biologic activity. ODNs may have sequence complementarity to important cellular genes, which inhibit gene translation.[11] These agents are commonly referred to as antisense ODNs and are currently undergoing extensive evaluation in the clinic.[12–14] Other ODNs have potent immunostimulatory activity determined by both the length and composition of the backbone and the sequence of deoxynucleotides. ODNs containing unmethylated CG dinucleotides are known as CpG ODNs, and have potent immunostimulatory effects.[15,16] In animal models, CpG ODNs have been found to have antitumor activity as single agents,[17,18] when used as an immune adjuvant in cancer vaccine strategies,[19] and in combination with

antilymphoma moabs.[19] In humans, CpG ODNs can increase expression of several interferon-inducible genes.[20]

The receptor for CpG ODN is the pattern receptor known as toll-like receptor 9 (TLR9), found in humans in B cells and plasmacytoid dendritic cells (PDCs).[16,21,22] CpG ODNs have potent direct stimulatory effects on B cells and dendritic cells. Both B cells and PDCs up-regulate class II MHC and the costimulatory molecules CD80 and CD86 in response to CpG ODN. CpG ODNs in select conditions also enhance expression of CD20, CD22, CD25, CD52, CD80, and HLA-DR on malignant human B cells, all of which are targets for therapeutic moabs.[23,24] CpG ODN induces production of a number of T_H1-like cytokines by PDCs, including IFN-α/β, IL-12, IP-10, and other T_H1-promoting cytokines and chemokines. NK cells are activated secondarily, secreting IFN-γ and gaining lytic activity.[15,25,26] The overall potential effect of these changes induced by CpG ODN is to enhance ADCC and NK-mediated lysis.

Because the mouse and human TLR9 molecules have different structures and different fine specificities,[27] a CpG ODN optimized for activation of the human TLR9 was developed.[28] This ODN, CPG 7909 (previously published as ODN 2006, and now known as PF-3512676), has a nuclease-resistant phosphorothioate backbone with 3 unmethylated CpG motifs, one of which contains 2 CpG dinucleotides. In preclinical toxicology studies, CPG 7909 seemed safe at doses up to 10 mg/kg in repeat dose toxicity studies using 4 weekly doses of IV bolus or 2-hour IV infusions. Like other phosphorothioate ODNs, administration of high-dose CPG 7909 induced a slight decline in platelet count, increased APTT, and complement activation.[29] CPG 7909 also has been tested for safety and immune activation in normal volunteers, through both the IV and the SC routes of administration at doses from 0.0025 to 0.32 mg/kg.[30]

On the basis of the potential importance of ADCC and NK-cell activity in patients with lymphoma, the immunologic activity demonstrated with CpG ODN in preclinical models, and the observed direct effect of CpG 7909 on the phenotype of primary lymphoma cells in vitro, we performed a disease-specific, phase I trial of single-agent CPG 7909 in patients with relapsed or refractory non-Hodgkin lymphoma. This study was designed to: (1) determine the safety and tolerability of CpG 7909 over a range of doses; (2) obtain preliminary information on the effect of CPG 7909 on ADCC, NK-cell activity, effector cell phenotype, and cytokine production, and; (3) assess for evidence of clinical activity.

PATIENTS AND METHODS

Patient Selection

Adult (age 18 y or greater) men and women with relapsed or refractory NHL who failed one or more therapies for NHL were eligible for the study. They were required to have an ECOG performance status of 0 to 2, measurable disease on radiologic imaging or physical examination, and adequate bone marrow and visceral organ function. Women of childbearing potential were required to have a negative serum β-hCG. Human Subjects Research Committees at each institution approved the study procedures, and all patients provided signed, informed consent. Patients were not allowed to have corticosteroids (60 mg or more of prednisone or equivalent) within 1 week or hematopoietic growth factors within 2 weeks of enrollment. Patients were not enrolled within 4 weeks of major surgery, radiotherapy, chemotherapy, or immunotherapy.

Patients were excluded if they had uncontrolled brain metastases, known preexisting autoimmune or antibody-mediated diseases, blood clotting disorders (including current therapy with anticoagulants other than aspirin), significant cardiovascular or pulmonary disease, or were positive for the human immunodeficiency virus.

Study Design

Dose ranges for the current study were based on animal toxicity data and prior studies of ODNs in humans. CPG 7909 was given once weekly as a 2-hour IV infusion for 3 consecutive weeks. A schedule of 3 weekly infusions was selected to be concordant with common schedules of clinical moab with the expectation that in subsequent studies, CpG 7909 would be started with the second infusion of moab to minimize overlap between the infusion-related toxicity seen with moab, that is generally most severe with the first infusion, and any infusion-related toxicity due to the study agent. Patients were dosed with CPG 7909 at 1 of 7 sequential dose levels including 0.01, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/kg (3 patients each). Subsequently, patients were enrolled at dose level 0.48 mg/kg (n = 5). Intrapatient doses were not adjusted during the course of treatment for fluctuations in weight. Dose-limiting toxicity (DLT) was defined as the presence of one or more of the following: (1) National Cancer Institute Clinical Toxicity Criteria (NCI/CTC version 2) Grade 4 nonhematologic toxicity sustained for any length of time; or (2) Grade 3 or 4 hematologic toxicity sustained for at least 5 weeks. Grade 4 nonhematologic toxicity was identified prospectively for this study due to risk of transient infusion-related toxicities that can easily meet NCI criteria for grade 3 toxicity.

Cohorts of 3 patients were evaluated at each dose level. If there was no evidence of dose-limiting toxicity in any patient 21 days after the final dose was administered, then the next dose level was initiated. If 1 patient experienced DLT, then 3 more patients were studied at that dose level. If more than 1 patient demonstrated DLT, dose escalation was halted and subsequent patients were studied at a dose level determined as the mean of the highest 2 previous cohorts studied.

Clinical Agent

CPG 7909 is a 24-mer tricosasodium salt phosphorothioate ODN of the following sequence:

5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′ dissolved in a phosphate-buffered saline solution without preservatives. Study supply of CPG 7909 was supplied by Coley Pharmaceutical Group (Wellesly MA) in 2 mL flint glass vials at a concentration of 20 mg/mL. Drug was stored upright at 2 to 8°C, protected from light, and was diluted at site in physiologic saline into clear glass containers at a final volume of 110 mL. One hundred mL was administered as a 2-hour infusion at a continuous rate of 50 mL/h once weekly for 3 doses. No premedication or other IV hydration was administered. Patients were hospitalized and monitored for 24 hours from the onset of each infusion.

Pretreatment and Follow-up Safety Studies

Patients were evaluated before, during, and after CPG 7909 therapy with history, examination, complete blood count, urinalysis, pulse oximetry, serum chemistries including glucose, renal and hepatic profiles, coagulation proteins including protime, activated partial thromboplastin time and fibrinogen, electrocardiogram recordings, and immunologic assays including erythrocyte sedimentation rate, antinuclear antibodies, anti-double–stranded DNA, C3, C4, and CH50. Patients were examined by the investigator before each infusion. Patients were housed overnight in the Clinical Research Center and monitored for 48 hours after each infusion. Blood samples for coagulation, immune activation, and complement activation assays were drawn immediately before and 2, 4, 6, 12, 24, and 48 hours after the first infusion and before and 2, 4, 24, and 48 hours after the second and third infusions. Hematology and serum chemistry samples were drawn immediately before and 2, 24, and 48 hours after each infusion. Blood samples were repeated for 1 and 4 weeks after the final infusion. A chest x-ray and a 12-lead EKG were obtained within 14 d of the first infusion and repeated 4 weeks after the final infusion.

Disease Evaluation

Tumor measurements were obtained by computed tomography (CT). Patients were evaluated for measurable disease with CT scans of low neck through pubis within 1 month of enrollment. Bone marrow was also sampled during this period if not known to be positive within the previous 180 days. Patients underwent CT scanning procedures again on day 42. Response was determined according to International workshop Criteria.[31] Evaluations after day 42 were at the discretion of the treating physician.

Pharmakokinetic and Cytokine Assays

Frozen serum samples were shipped on dry ice. A temperature monitor was included with each shipment to ensure that the samples remained frozen during shipment. Upon receipt, sample identity was verified and sample condition assessed. Samples were then transferred to −80°C for storage until analysis. The quantification of CPG 7909 in human EDTA plasma was conducted by Coley Pharmaceutical Group with a colorimetric hybridization assay, using capture and detection oligonucleotides complementary to portions of the sequence of CPG 7909. The linear range of the assay is 7.8 to 1000 pg/mL with a lower limit of quantification of 7.8 pg/mL and a detection limit of 2.8 pg/mL.

Commercial enzyme-linked immunosorbant assay kits were used for quantification of serum IL-6 (HS600), IL-12p40 (DP400), IL-18 (7620), TNF-α (HSTA00C), MCP-1 (DCP00), and MIP-1β (DMB00), all purchased from R&D Systems, Minneapolis, MN, and C-RP (030-9710 s) purchased from Alpco Diagnostics, Windham, NH. All assays were performed according to the manufacturer's specifications.

An IP-10 enzyme-linked immunosorbant assay was developed to assess serum IP-10 levels. Briefly, 96-well plates were coated with 0.5 μg/mL of mouse antihuman IP-10 (BD Biosciences Pharmingen, San Diego, CA, 23161D). After a blocking step, the plates were washed and IP-10 standards (Human IP-10, BD Biosciences Pharmingen, 24541V) ranging in concentration from 19.5 to 2500 pg/mL were added as were serial dilutions of the test serum. After incubation, secondary antibody, biotinylated mouse antihuman IP-10 (BD Biosciences Pharmingen, 23172D), was added, followed after incubation with avidin peroxidase (Sigma-Aldrich, Inc, A3151). After washing, a colorimetric substrate was added. Absorbance readings for each well were determined. Samples that were below the lowest standard were considered out of range and recorded as less than the lowest standard times the dilution factor. Samples that had absorbance readings higher than that of the highest standard were further diluted as required and reassessed as outlined above.

Serum Ig levels were evaluated by adding serial dilutions of serum to plates coated with mouse antihuman IgM IgG Biotin-labeled antihuman IgG or IgM was then added followed after washing by a colorimetric substrate and evaluation on a microplate reader. Standard curves were established using samples with known Ig concentrations. Samples were assessed in at least duplicate at different times and further dilutions were prepared as required. Samples were also evaluated for monoclonal protein using immunofixation electrophoresis.

Immunologic Assays

NK activity was determined using fresh, autologous peripheral blood mononuclear cells. These cells were assayed for their ability to kill K562 target cells in a standard 4-hour ^{51}Cr-release assay. Lysis was examined in wells to which IL-2 (100 U/mL) was added at the beginning of the assay.

ADCC is not species specific. Therefore, ADCC was determined using the NK-resistant CL27A murine lymphoma, which lacks human B-cell antigens and has been maintained in our laboratory for over 15 years for ADCC assays. Fresh, peripheral blood mononuclear cells obtained from subjects were added to ^{51}Cr-labeled CL27A cells. Polyclonal anti-CL27A lymphoma antibody

10 μg/mL, obtained from hyperimmunized mice, was used as the source of antibody. Lysis was evaluated after 4 hours. All assays were performed in duplicate with freshly isolated PBMC. PBMC effector activity was expressed in Lytic Units, and all values are presented as referenced to pretreatment values.

Three and 4 color flow cytometric analysis of PBMCs using standard gating parameters was performed to assess numbers of NK, B, and T cells. Some subjects had insufficient cell numbers for both functional and phenotypic analyses. In such cases, only the functional analyses were performed.

RESULTS

Patients

Twenty-three patients received 67 infusions of CPG 7909 at doses ranging from 0.01 to 0.64 mg/kg/w. One patient gave additional history of previous diagnosis of multiple sclerosis (judged a disqualifying autoimmune-related diagnosis) when presenting for the second infusion and was removed from study without further treatment. Twenty-three patients are evaluable for safety and response and 21 for immunologic changes. Patient characteristics (Table 1) are notable for median age of 63 years, a diversity of indolent and aggressive lymphoma histologies, generally advanced-stage disease and good performance status at time of enrollment. The median number of prior treatments was 4. Three patients were enrolled at each dose level up through a dose level of 0.64 mg/kg. Five patients were subsequently enrolled at a dose level of 0.48 to gain further data regarding safety and immunologic activity at that dose level.

Toxicity

All patients were assessable for toxicity. The regimen was well tolerated with infrequent transient grade 1 and 2 adverse events, including hyperglycemia, nausea, chills/rigors, hypotension, and fever. No patient discontinued therapy secondary to toxicity. All observed adverse events are summarized in Table 2. Serious adverse hematologic events observed more than once include anemia (2 = Gr3, 2 = Gr4), thrombocytopenia (4 = Gr3), and neutropenia (2 = Gr3), and were largely judged because of progressive disease. Six patients developed transient lymphopenia, with a median time to lymphocyte recovery of 48 hours or less. Two subjects at a dose level of 0.64 mg/kg had grade 4 anemia that fulfilled protocol defined criteria for dose deescalation. Subsequent evaluation of these 2 subjects suggested that the experimental agent was not likely responsible for anemia. In 1 case, the subject entered the study with significant marrow infiltration with lymphoma and developed transfusion dependence in the month after treatment. Further marrow studies in this subject revealed progressive lymphoma infiltration with virtual replacement of normal hematopoietic precursors. In the second case, transfusion dependence developed within 1 month after experimental treatment. Marrow evaluations revealed pancytopenia with chromosomal mutation and deletion of 7q. This patient was previously treated with cyclophosphamide, and subsequent evaluation of preenrollment marrow samples revealed that the 7q abnormality existed before enrollment, indicating a previously unrecognized myelodysplasia. Two patients developed grade 3 dyspnea judged secondary to progressive lymphoma. Toxicities temporally related to the infusion were minimal and limited to grade 1 nausea, hypotension, and intravenous catheter discomfort. Five patients were noted to develop proteinuria (3 = Gr1, 2 = Gr2). One patient at dose level 0.01 mg/kg developed transient prolongation of aPTT, and no patient developed a prolonged protime.

TABLE 1. Characteristics of Patients Enrolled and Treated

Characteristics at Entry	No. Subjects
No patients	23
Median age (range)	63 (36 to 79)
Sex	
Male	14
Female	9
Histology	
Diffuse large B cell	7
Follicular	8
Small lymphocytic	1
Peripheral T cell	1
Mantle cell	2
Other	4
Prior chemotherapy courses, median (range)	4 (1 to 9)
Stage	
I/II	9
III/IV	14
Performance status (WHO)	
0	14
1	6
2	3

TABLE 2. Observed Adverse Events (n = 23)*

	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	3	1	1	2
Thrombocytopenia	1		4	
Neutropenia	4	4	2	
Lymphopenia	1	2	3	
Dyspnea	1	4	2	
Chills/rigors	1	1		
Nausea/vomit	7	1		
Fever	3			
Hypotension	6	1		
Fatigue	2	3	1	
Pain	6	2		
Venous/site rxn	6			
aPTT prolongation		1		
Hypocalcemia	2	2		
CO_2 depression	5			
ALT elevation	2		1	
Hyperglycemia	9	4		
Proteinuria	3	2		

*All toxicities grade ≥ 3 and of any grade occurring in ≥ 2 subjects.

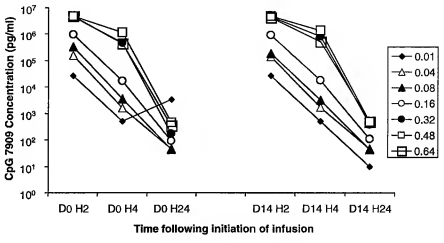

FIGURE 1. Serum drug levels of CPG 7909 at 2, 4, and 24 hours after initiation of a 2-hour IV infusion. Data for the first and third infusions, grouped by dose level (mg/kg), are shown.

No evidence of complement activation was observed in serum complement assays.

CPG 7909 Pharmacokinetics

All patients exhibited detectable CPG 7909 in the serum immediately after conclusion of each 2-hour infusion (Fig. 1). Mean postinfusion levels ranged from 23 ng/mL for patients dosed at 0.01 mg/kg to 4.8 μg/mL for patients dosed at 0.64 mg/kg. Clearance of drug was rapid with a 5 to 50-fold decrease in detectable serum CPG 7909 within 2 hours after the completion of the infusion. Serum drug kinetics was similar after the first (D0) and third (D14) infusions.

Quantitative NK-Cell Changes

NK cells ($CD56^+$, $CD3^-$) were increased in absolute numbers beginning at day 2 and continuing to day 42 in most, but not all subjects (Fig. 2A). In comparing pretherapy to posttherapy NK numbers (Fig. 2B), the mean ratio of NK-cell concentrations at day 2 was 1.44 (95% CI = 0.94–1.94) and at day 42 was 1.53 (95% CI = 1.14-1.91). A dose-dependent increase in NK-cell number was not evident when analyzed in aggregate or at day 42. No consistent changes in numbers of B or T cells were noted after therapy (data not shown).

Qualitative Changes in NK Activity and ADCC

NK activity of fresh PBMCs measured against K562 cells generally increased in subjects compared with pretreatment values (Fig. 3). Effector-to-target ratios were based on total PBMC, and not NK-cell numbers, because NK-cell numbers are not the only determinant of NK activity and ADCC. The increase in NK activity was most apparent at the lower dose levels studied and generally was evident from the third week and frequently persisted at evaluation 4 weeks after the last infusion, consistent with the quantitative changes seen in NK cells. Subject 11 demonstrated the greatest change in NK activity in response to CPG 7909. This subject had a low lymphocyte count both before and after therapy, which prevented phenotypic analysis of the PBMCs in this particular case. ADCC was assessed with patient PBMCs against the NK-resistant CL27A murine lymphoma, which lacks human B-cell antigens. Human effector cells can use murine antibody to kill a murine target. Therefore, this assay allowed for evaluation of ADCC independent of any antibody in the subjects' circulation. ADCC activity in several cohorts was relatively unchanged over the course of therapy and follow-up (Fig. 4). Of notable exception, patients treated with 0.16 mg/kg demonstrated increased ADCC function measured during the third week of infusions and persisting for at least 1 week thereafter.

Serum Cytokine Changes

Serum levels of IL-6 rose transiently after the first injection, peaking at 6 to 12 hours (mean peak ratios = 3.75; range 2.4 to 5.5) and returned to baseline by 48 hours. There were no biologically significant changes in other measured serum cytokines (IL-12, IL-18, TNF-α), chemokines (IP-10, MCP-1, MIP-1b), or markers of immune activation (IgM, IgG, C-reactive protein) at any of the dose levels tested.

Serum Immunoglobulins

CpG ODN is known to enhance Ig production by benign B cells. We, therefore, evaluated for quantitative changes in serum immunoglobulins and for the appearance of monoclonal gammopathies by evaluating serum Ig obtained before and after CpG ODN therapy in 21 subjects with available serum. Quantitative heavy-chain levels in all subjects were within or mildly below normal limits and none changed post-CpG ODN treatments by more than 20% of baseline (data not shown). Among those with $> 5\%$ but $\leq 20\%$ change from baseline, there were 5 subjects with increased levels and 5 with decreased levels. Two of 3 subjects with preexisting monoclonal IgM serum paraproteins had a slight increase in IgM

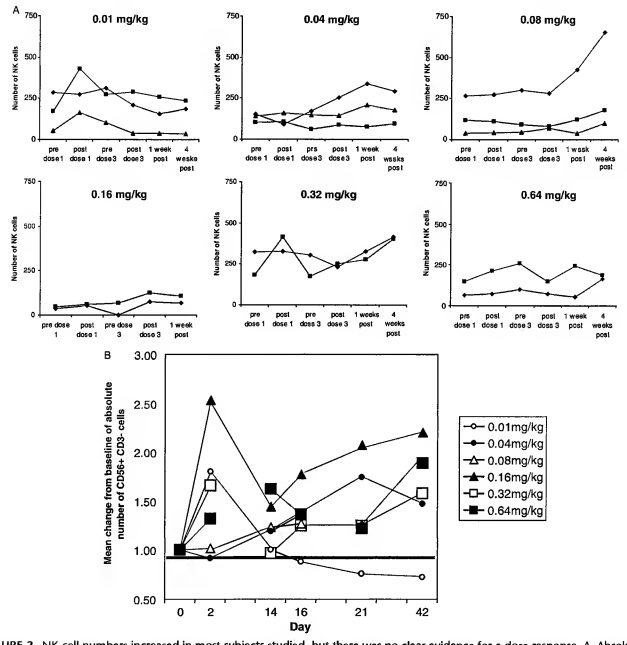

FIGURE 2. NK-cell numbers increased in most subjects studied, but there was no clear evidence for a dose response. A, Absolute numbers of NK cells in subjects treated at different dose levels. B, Mean NK-cell concentrations for each of the first six dose levels at described time points after treatment with CPG 7909. All values are presented as referenced to pretreatment values. The mean relative number of NK cells compared with pretreatment at day 2 was 1.44 and at day 42 was 1.53.

paraprotein and the other had a decrease. No new monoclonal paraproteins were identified.

Antitumor Activity

The only formal assessment of response occurred on day 42, at which time no patient met NCI consensus criteria for response. Two patients subsequently developed radiographic evidence of disease regression without further therapy, and met International Workshop Criteria for Complete Response[31] (Table 3). Both subjects had low disease burden at time of study enrollment. Patient 0102, treated at a dose level of 0.01 mg/kg had an isolated submandibular node, which was biopsy proven recurrent follicular lymphoma at study entry. Serial CT scans (Fig. 5) demonstrated resolution of this node beginning 3 months after receiving study drug, and the patient

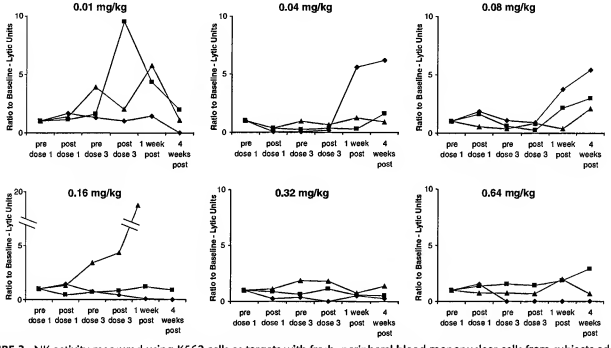

FIGURE 3. NK activity measured using K562 cells as targets with fresh, peripheral blood mononuclear cells from subjects added. NK-effector activity is expressed in Lytic Units, and all values are presented as referenced to pretreatment values.

is without clinical or CT evidence of disease after 38 months of follow-up. Patient 0102 is of special interest because he also demonstrated a marked increase in $CD56^+/CD3^-$ cells in the periphery after CpG 7909 administration (Fig. 6). Patient 0211, treated at dose level 0.16 mg/kg, had a solitary pulmonary nodule known to be

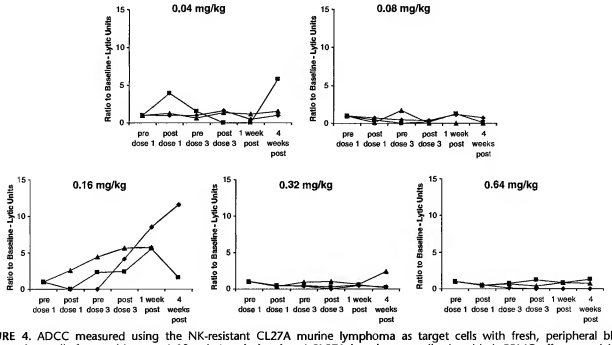

FIGURE 4. ADCC measured using the NK-resistant CL27A murine lymphoma as target cells with fresh, peripheral blood mononuclear cells from subjects and 10 μg/mL polyclonal anti-CL27A lymphoma antibody added. PBMC effector activity is expressed in Lytic Units, and all values are presented as referenced to pretreatment values.

TABLE 3. Tumor Measurements (in mm) in Late Responding Subjects

	Baseline	Day 42	Wk 18	Wk 30 to 34
Patient 0102	30 × 20	28 × 25	18 × 15	12 × 8
Patient 0211	18 × 10	18 × 10	N/A	Undetectable

recurrent diffuse large B-cell lymphoma by biopsy at study entry. Serial CT scans demonstrated no change in the nodule at day 42; however, subsequent CTs revealed resolution of this nodule 6 months after study drug. The patient developed progression in the lung 15 months later.

DISCUSSION

Our study demonstrates the safety of intravenous CPG ODN in patients and is the first published demonstration of a CPG ODN mediated increase in NK cells in previously treated cancer patients. The objectives of this study were to establish the safety of intravenous CPG 7909 infusions in previously treated lymphoma patients over a variety of dose ranges, and to observe for any measurable dose-related change in the effector cell functions in this potentially immunocompromised population. Selection of the weekly × 3 infusion schedule was based on the anticipated coupling of CPG 7909 with common schedules of therapeutic moabs.

Toxicity with ODNs can be both dependent and independent of specific base sequence.[32,33] In rodents, the immunostimulatory effect of CpG ODNs on B-cell proliferation manifests as splenomegaly and adenopathy.[34,35] No sequence-specific toxicity was observed in nonhuman primates with CpG ODNs after IV infusion; however, non–sequence-specific toxicity was observed. Blood levels of phosphorothioate ODNs above 40 to 50 μg/mL can interact with blood proteins causing idiosyncratic activation of the alternative complement pathway and inhibition of intrinsic coagulation mechanisms. Other organ manifestations are dependent on the dose level and frequency of administration but are consistent with the known distribution of ODNs to liver, spleen, kidney, and bone marrow.[36,37]

The dosing and safety of CPG 7909 has been studied in normal volunteers given 1 to 3 doses by the IV or SC routes in doses ranging from 0.0025 to 0.64 mg/kg.[30] In a study of subcutaneous dosing (2 injections separated by 14 d in doses ranging from 0.0025 to 0.08 mg/kg) in healthy male volunteers, adverse events were virtually universal but mild and included injection site inflammation, and flulike symptoms with increasing frequency associated with rising dose levels at or above 0.01 mg/kg. Several patients were noted to have a transient neutrophilia at 24 to 48 hours posttherapy followed by a transient grade 2 to 3 neutropenia resolving between days 7 and 15, suggesting a short-term compartment redistribution. In this study of normal volunteers, SC CPG 7909 also induced a T_H1-like pattern of systemic innate immune activation manifested by increased levels of IL-6, IL-12p40, IFN-α, and IFN-inducible chemokines. In contrast, in a similar study of intravenous dosing (2 injections separated by 7 d in doses ranging 0.001 to 0.32 mg/kg) in healthy male volunteers, local injection site reactions were rare, although systemic flulike reactions were described as mild and were reported with equivalent frequency in a placebo cohort. No perturbations in laboratory parameters including circulating neutrophils, cytokines, or chemokines were detected. Before initiation of the current study, there was much discussion of whether the CpG 7909 should be studied with administration intravenously or subcutaneously. The intravenous route in the current study was chosen on the basis of the preclinical models demonstrating augmentation of moab antitumor effect after systemic bolus administration.[19]

In the current study, using a 2-hour IV infusion of doses up to 0.64 mg/kg in patients with previously treated NHL, acute toxicity was minimal and consistent with the observations of intravenous infusions in normal volunteers.[30] The absence of evidence of complement activation

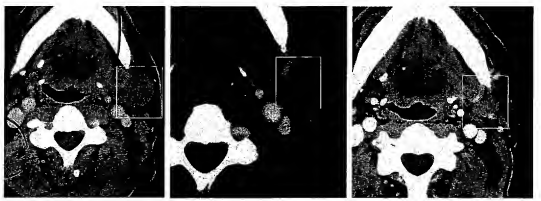

FIGURE 5. Serial CT scan images from a single subject demonstrate slow resolution of a left submandibular node beginning 3 months after receiving study drug.

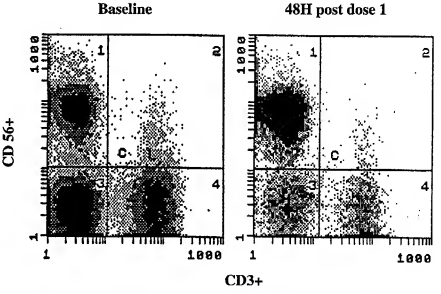

FIGURE 6. Flow histograms pre- and poststudy drug treatment of PBMCs from patient 0102 demonstrated a marked increase in $CD56^+/CD3^-$ cells in the periphery after CpG 7909 administration. Gates were set on lymphocytes as determined by forward versus side scatter on CD45-positive cells.

or coagulation abnormalities is consistent with the nonhuman primate studies given the low-peak serum concentrations of 0.000023 to 4.8 μg/mL of ODNs. These findings are similar to those of Friedberg et al[20] when studying the CPG ODN 1018 ISS in 4 weekly subcutaneous doses up to 0.5 mg/kg.

In vitro and animal modeling suggests immunostimulatory CpG ODN, such as CPG 7909, could mediate regression of lymphoma by a number of mechanisms. First, CpG ODN can enhance killing secondary to NK-cell activation.[18] Thus, the secondary objective of this study was to observe for measurable change in the effector cell functions. Patients in our study demonstrated an increase in measured NK-cell number and activity at multiple time points after receiving CPG 7909 compared with baseline. We found that change in NK numbers and in NK activity did not always correlate, suggesting future studies evaluating the effect of CpG ODN on both the growth and activity of various NK subsets could be worthwhile. Although the patients demonstrating the greatest relative increase are generally in the lower dose ranges studied, there are not enough subjects to make definitive conclusions regarding dose as a variable. Similarly, variables such as tumor burden, extent of prior immunosuppressive therapy, age, and lymphoma subtype did not clearly correlate with the observed immunologic effect. Despite rapid clearance of the CpG 7909 from the circulation, we noted prolonged immunologic effects as indicated by an expansion in NK cells and in some cases an increase in functional activity as measured by NK-mediated lysis and ADCC. This long-term change after short-term exposure to CpG ODN is consistent with observations in animal models, and is in marked contrast to the expected effects of antisense ODN, which seem to require prolonged exposure such as that resulting from a continuous infusion. Other immunologic adjuvants such as IL-2 have also demonstrated expansion in NK-cell populations in lymphoma patients and in studies combining IL-2 with rituximab, the NK cells were more numerous in responding subjects.[38]

Another mechanisms by which CpG ODN could mediate lymphoma regression is direct activation-induced cell death. We saw no evidence for rapid clinical response to CPG 7909 as might be expected from activation-induced cell death. One concern leading into this phase I trial was that CpG 7909 might actually induce proliferation or apoptosis resistance of malignant B cells, as it does benign B cells.[16] This effect has been less clear in preclinical studies with malignant B cells.[23] A number of subjects in the current study experienced progression of their lymphoma on CPG 7909 therapy, but these were subjects with disease that was progressing at the time of enrollment, and there was no observed acceleration in disease progression. No subjects with relatively quiescent disease before enrollment had rapid growth of tumor after exposure to CPG 7909. CpG ODN also induces benign B cells to secrete immunoglobulin.[16] Careful evaluation of serum immunoglobulins in the serum obtained after CpG ODN therapy in this trial demonstrated no evidence for an increase in detectable polyclonal immunoglobulins or detectable monoclonal gammopathy after CpG ODN therapy, even in those subjects with small monoclonal spikes before therapy. Thus, the direct, short-term effect of single-agent CPG 7909 on disease progression, disease regression, or immunoglobulin production by the malignant clone, seems to be limited.

We now know that CpG ODNs are not all alike.[18,25,39] A-Class CpG ODNs have a primary effect of inducing IFNa production by PDCs and secondarily activate NK cells, but have little direct effect on B cells. In contrast, B-Class CpG ODNs, such as the CPG 7909,

have broader immunologic effects. They activate B cells and plasmacytoid DC directly, but in most models induce much less IFN-α production as a result of which the secondary activation of NK cells is less pronounced than that seen with A-Class CpG. Interestingly, 1018 ISS—a B-Class CpG ODN studied in humans up-regulated expression of inducible genes in a pattern to suggest much of the biologic activity in vivo is mediated by type I IFNs.[20] Recently, several groups of investigators have described C-Class CpG ODNs, which have intermediate effects between the A-Class and B-Class, inducing higher levels of IFN-α secretion compared with the B-Class, but are weaker than A-Class.[40–42] As a B-Class ODN, CPG 7909 therefore would be expected to directly stimulate the TLR9-expressing NHL cells to up-regulate costimulatory molecule expression, and expression of CD20, which should make them more immunogenic. However, it could be argued that A or C-Class CpG would be more attractive to combine with moabs given the growing evidence that NK cells play a central role in mediating the antitumor activity of such antibodies. In preclinical models with B-Class ODNs, we found both PMN and NK cells contribute to optimal ADCC.[25] The in vitro ADCC assay used in the current study did not include PMN which may, in part, explain our inability to detect consistent ADCC across dose levels and in future studies it would be desirable to perform these assays using whole blood.

Moabs have significant antitumor activity in a number of human tumors, most notably lymphoma. Although moabs are well tolerated, their antitumor activity even in the best of circumstances is variable, incomplete, and transient. Several lines of investigation are underway to build upon the successes to date including the use of adjuvant therapies designed to augment the immunotherapeutic potential of moabs. These have been most extensively evaluated in lymphoma settings and include the addition of IFN, granulocyte macrophage-colony stimulating factor, IL-2, and IL-12 to moab.[43–46] Each of these approaches is predicated on the attempt to augment the potency of effector cells thought to be important in moab therapy. Ultimate success in this line of investigation will depend upon the ability to harness and augment the therapeutic attributes of immune activation while minimizing the toxic attributes. The apparent lack of significant toxicity, and some evidence for enhanced NK-cell numbers, NK activity, ADCC, makes the combination of CpG ODN and moab an attractive approach to trying to enhance the efficacy of moab therapy.

In summary, preclinical evaluation suggests that CpG ODNs enhance NK-cell activity and ADCC, increase expression of costimulatory molecules by the malignant B cells and increase expression of CD20. The phase I trial presented here is the first to demonstrate changes in NK populations and to a lesser extent ADCC in patients with cancer and confirms that CpG 7909 can be delivered safely in patients with lymphoma as a 2-hour infusion. Subjects studied at doses of 0.64 mg/kg or higher should be observed carefully for hematologic toxicity. Future dose finding studies of CpG 7909 or similar agents should include attention to NK-cell number and activity as a measure of biologic activity. Further studies are necessary to determine whether immune stimulation, alone or in combination with other treatments, will have beneficial therapeutic effects for patients.

REFERENCES

1. Portlock CS. Alpha-interferon in the treatment of non-Hodgkin's lymphomas. *Curr Concepts Oncol.* 1987;9:2–6.
2. McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol.* 1998;16:2825–2833.
3. Redman BG, Flaherty L, Chou TH, et al. phase I trial of recombinant interleukin-2 combined with recombinant interferon-gamma in patients with cancer. *J Clin Oncol.* 1990;8:1269-1276.
4. Duggan DB, Santarelli MT, Zamkoff K, et al. A phase II study of recombinant interleukin-2 with or without recombinant interferon-beta in non-Hodgkin's lymphoma. A study of the Cancer Leukemia Group B. *J Immunother.* 1992;12:115–122.
5. Golay J, Zaffaroni L, Vaccari T, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 CD59 regulate complement-mediated cell lysis. *Blood.* 2000;95:3900–3908.
6. Harjunpaa A, Junnikkala S, Meri S. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. *Scand J Immunol.* 2000;51:634–641.
7. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood.* 1994;83:435–445.
8. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood.* 2002;99:754–758.
9. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol.* 2003;21:3940–3947.
10. Bohen SP, Troyanskaya OG, Alter O, et al. Variation in gene expression patterns in follicular lymphoma the response to rituximab. *Proc Natl Acad Sci U S A.* 2003;100:1926–1930.
11. Agarwal N, Gewirtz AM. Oligonucleotide therapeutics for hematologic disorders. *Biochim Biophys Acta.* 1999;1489:85–96.
12. Frankel SR. Oblimersen sodium (G3139 Bcl-2 antisense oligonucleotide) therapy in Waldenstrom's macroglobulinemia: a targeted approach to enhance apoptosis. *Semin Oncol.* 2003;30:300–304.
13. Waters JS, Webb A, Cunningham D, et al. Phase I clinical pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol.* 2000; 18:1812–1823.
14. Williams SA, Gillan ER, Knoppel E, et al. Effects of phosphodiester phosphorothioate antisense oligodeoxynucleotides on cell lines which overexpress c-myc: implications for the treatment of Burkitt's lymphoma. *Ann Oncol.* 1997;8 (Suppl 1):25–30.
15. Krieg AM. CpG motifs in bacterial DNA their immune effects. *Annu Rev Immunol.* 2002;20:709-760.
16. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* 1995;374:546–549.
17. Heckelsmiller K, Rall K, Beck S, et al. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J Immunol.* 2002;169:3892–3899.
18. Ballas ZK, Krieg AM, Warren T, et al. Divergent therapeutic immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J Immunol.* 2001;167:4878–4886.
19. Wooldridge JE, Ballas Z, Krieg AM, et al. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood.* 1997; 89:2994–2998.

20. Friedberg JW, Kim H, McCauley M, et al. Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-alpha/beta-inducible gene expression, without significant toxicity. *Blood.* 2005;105:489–495.
21. Kemp TJ, Elzey BD, Griffith TS. Plasmacytoid dendritic cell-derived IFN-alpha induces TNF-related apoptosis-inducing ligand/Apo-2L-mediated antitumor activity by human monocytes following CpG oligodeoxynucleotide stimulation. *J Immunol.* 2003;171: 212–218.
22. Latz E, Schoenemeyer A, Visintin A, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol.* 2004;5:190–198.
23. Jahrsdorfer B, Hartmann G, Racila E, et al. CpG DNA increases primary malignant B cell expression of costimulatory molecules target antigens. *J Leukoc Biol.* 2001;69:81–88.
24. Decker T, Schneller F, Sparwasser T, et al. Immunostimulatory CpG-oligonucleotides cause proliferation cytokine production, an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood.* 2000;95:999–1006.
25. van Ojik HH, Bevaart L, Dahle CE, et al. CpG-CpA B oligodeoxynucleotides enhance the efficacy of antibody therapy by activating different effector cell populations. *Cancer Res.* 2003;63: 5595–5600.
26. Krieg AM. CpG motifs: the active ingredient in bacterial extracts? *Nat Med.* 2003;9:831–835.
27. Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A.* 2001;98:9237–9242.
28. Hartmann G, Weeratna RD, Ballas ZK, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro in vivo. *J Immunol.* 2000;164: 1617–1624.
29. Levin A, Henry S, Monteith D, et al. Toxicity of antisense oligonucleotides. In: Crooke ST, ed. *Antisense Drug Technology: Principles, Strategies, and Applications.* New York: Marcel Dekker; 2001:201–267.
30. Krieg AM, Efler SM, Wittpoth M, et al. Induction of Systemic TH1-Like Innate Immunity in Normal Volunteers Following Subcutaneous but Not Intravenous Administration of CPG 7909 a Synthetic B-Class CpG Oligodeoxynucleotide TLR9 Agonist. *J Immunother.* 2004;27:460–471.
31. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J Clin Oncol.* 1999;17:1244.
32. Monteith DK, Levin AA. Synthetic oligonucleotides: the development of antisense therapeutics. *Toxicol Pathol.* 1999;27:8–13.
33. Monteith DK, Geary RS, Leeds JM, et al. Preclinical evaluation of the effects of a novel antisense compound targeting C-raf kinase in mice monkeys. *Toxicol Sci.* 46:365–375.
34. Sarmiento UM, Perez JR, Becker JM, et al. In vivo toxicological effects of rel A antisense phosphorothioates in CD-1 mice. *Antisense Res Dev.* 1994;4:99–107.
35. Monteith DK, Henry SP, Howard RB, et al. Immune stimulation—a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des.* 1997;12:421–432.
36. Henry SP, Templin MV, Gillett N, et al. Correlation of toxicity pharmacokinetic properties of a phosphorothioate oligonucleotide designed to inhibit ICAM-1. *Toxicol Pathol.* 1999;27:95–100.
37. Henry SP, Beattie G, Yeh G, et al. Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int Immunopharmacol.* 2002;2:1657–1666.
38. Gluck WL, Hurst D, Yuen A, et al. Phase I studies of interleukin (IL)-2 and rituximab in B-cell non-hodgkin's lymphoma: IL-2 mediated natural killer cell expansion correlations with clinical response. *Clin Cancer Res.* 2004;10:2253–2264.
39. Verthelyi D, Ishii K, Gursel M, et al. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J Immunol.* 2001;166:2372–2377.
40. Vollmer J, Weeratna R, Payette P, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol.* 2004;34:251–262.
41. Hartmann G, Battiany J, Poeck H, et al. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur J Immunol.* 2003;33:1633–1641.
42. Marshall JD, Fearon K, Abbate C, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol.* 2003;73: 781–792.
43. Kimby E. Beyond immunochemotherapy: combinations of rituximab with cytokines interferon-alpha2a and granulocyte colony stimulating factor [corrected]. *Semin Oncol.* 2002;29:7–10.
44. Ansell SM, Witzig TE, Kurtin PJ, et al. Phase 1 study of interleukin-12 in combination with rituximab in patients with B-cell non-Hodgkin lymphoma. *Blood.* 2002;99:67–74.
45. Davis TA, Maloney DG, Grillo-Lopez AJ, et al. Combination immunotherapy of relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma with rituximab and interferon-alpha-2a. *Clin Cancer Res.* 2000;6:2644–2652.
46. Friedberg JW, Neuberg D, Gribben JG, et al. Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. *Br J Haematol.* 2002;117:828–834.